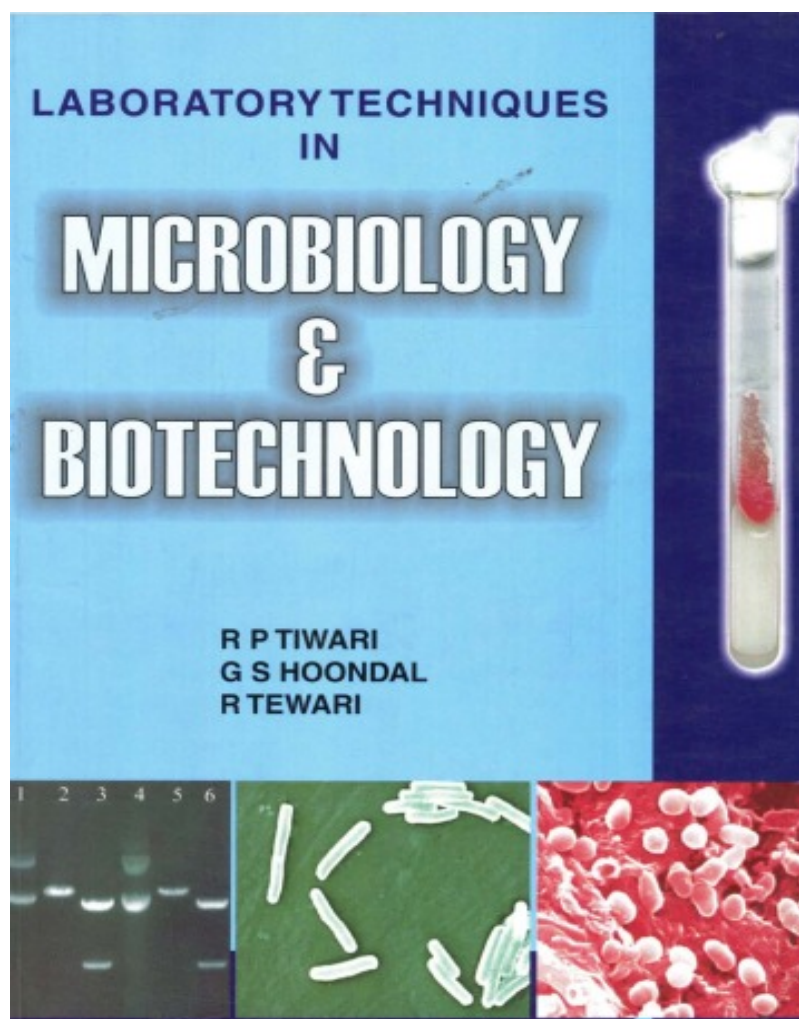


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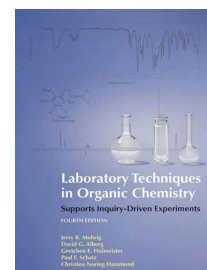


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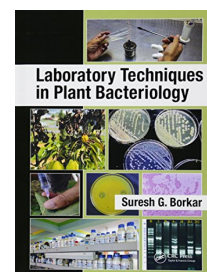
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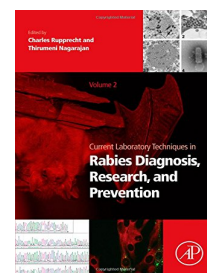
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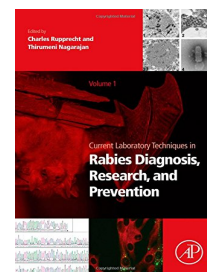
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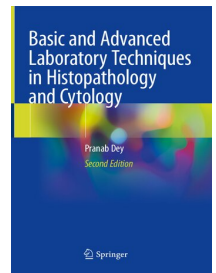
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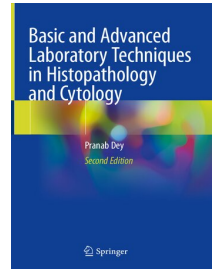
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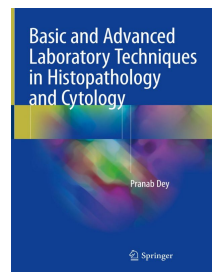
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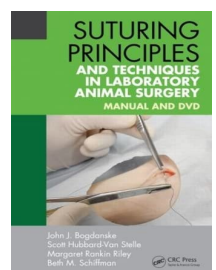
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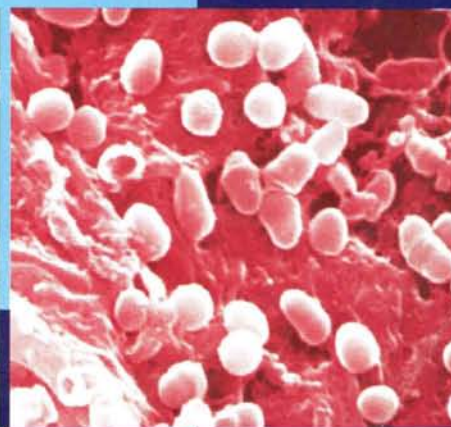
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**LABORATORY TECHNIQUES
IN**

MICROBIOLOGY & BIOTECHNOLOGY

**R P TIWARI
G S HOONDAL
R TEWARI**



**LABORATORY TECHNIQUES
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&
BIOTECHNOLOGY**

■ R. P. TIWARI ■ G. S. HOONDAL ■ R. TEWARI

Departments of Microbiology and
Biotechnology, Panjab University, Chandigarh



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Preface

The present manual comes from the teachers who have more than twenty-five years experience of teaching and research in microbiology and biotechnology to undergraduate and postgraduate classes. The manual has been written for University undergraduates (Medical and Engineering streams) having interest in Microbiology and Biotechnology. Students joining these disciplines are primarily the science students who may have only a brief exposure to microbiology being taught as small component along with biology. Much emphasis is on the exercises in botany and zoology and that through models. Some students from non-medical stream who even lack the basics of biology or have forgotten what they have learnt up to matriculation join B.E Biotechnology and Food Processing Technology courses. Students often lack the knowledge about the handling of microbes despite the fact they have the information about their potential as foes and friends. Hence it becomes obligatory to impart them good training comprising basics of biosciences, microbiology and biotechnology at least at the university or college level to comprehend the concept of sterile techniques, cross contamination, decontamination in laboratory, aseptic transfer techniques form one culture medium to another especially during cultivation and examination of microbial and cellular forms.

Although there has been a sea change in the research activities with the advent of, molecular biology and biotechnology but it is not possible to make progress unless one is conversant with the microbiological techniques pertaining to handling of microbes which has not changed over years. In recent years, very few books have been published pertaining to basic techniques, which have been neglected because of hype created with the emergence of relatively newer branches like biotechnology that also require one to efficient and proficient in handling of microbes to be a good biotechnologist.

We have the pleasure in bringing about the manual of techniques in microbiology and biotechnology that would be highly useful for the students of biosciences, microbiology and biotechnology. The manual has been divided in six sections pertaining to exercises in GENERAL MICROBIOLOGY, APPLIED MICROBIOLOGY, BACTERIAL PHYSIOLOGY, MEDICAL MICROBIOLOGY, IMMUNOLOGY, BACTERIAL GENETICS AND BIOTECHNOLOGY. The experiments have been given in chronological orders and most of these can be performed with materials available in most biological sciences laboratories. For some of the experiments even alternate procedures have been suggested. The experiments covered in this manual are easy to perform, and have been used over the years in our laboratory for imparting practical training regularly to undergraduates and postgraduate students and summer trainees. In the beginning of each experiment, a brief introduction of the exercise familiarizes the students about the nature of experiment and its objective. It is followed by a detailed stepwise protocol. Exercises have been explained in easy and explicit language. Suggestions from readers and users are welcome for its improvement in future (Email: ramptiwari@yahoo.com).

Authors

Introduction

Microbiology and biotechnology are not mere facts, terms, and concepts. In the present era of biological research, these two applied subjects are making significant contributions towards scientific knowledge and solving human problems. In order to be an accomplished biotechnologist, a thorough knowledge of Microbiology is essential.

Microorganisms were probably the first living forms to appear on the earth. Microorganisms are omnipresent in biosphere. Most microorganisms are free living and perform useful activities. Biosphere in general comprises of two categories: animate or living and inanimate or non-living beings. Biology is the science that deals with the living organisms. The branch of biology that deals with microscopic forms of living organisms (microbes) is termed as the “microbiology”.

The emergence and development of microbiology has been highly erratic in the beginning. Credit for introduction to microbial world goes to Antony van Leeuwenhoek and to vistas of microbial activities to Louis Pasteur and Robert Koch. This period is known as the “Golden Era” of microbiology because of the significant discoveries on various aspects of microbiology: microbial fermentations, discovery of various disease causing agents, microbes cultivations, differences in metabolic activities, development of attenuated strains and their use as immunoprophylactic agents.

Microbial kingdom includes the study of bacteria, rickettsiae, viruses, fungi, algae and protozoa. Microbiology is further subdivided into bacteriology dealing with bacteria and rickettsiae, virology the study of viruses, mycology study of fungi and protozoology deals with protozoa. Microbiology considers the microscopic forms of life as to their occurrence in nature, their reproduction and physiology, their harmful and beneficial relationship with other living things and their significance in science and industry. Microbiologists study bacteria in many ways. The methods used to study bacteria and other microbes include direct microscopic examination, cultivation, biochemical tests, animal inoculation, serological reactivity and recent molecular biological techniques. Currently because of their high versatility and rapid turn over microorganisms are used extensively in genetic engineering and biotechnological processes and new processes are being developed to produce variety of enzymes of industrial importance, vaccines, insecticides, pharmaceuticals and other biological products of interest to mankind, animals and plants.

Laboratory Safety Rules

1. Always, mop the bench with disinfectant such as 2% phenol or polysan before and after use.
2. Always, wash your hands thoroughly with soap and water at the beginning and at the end of each laboratory period.
3. Keep windows and doors closed to reduce air borne contamination.
4. Be systematic and logical. Keep a faithful record of all the experiments and observations. Update it regularly and submit it for evaluation at the end of each exercise.
5. Always, wear overcoat/apron while working in laboratory. It should be washed at least once a week. Keep the hairs and loose garments under check.
6. Always, wear gloves if there are cuts on hands or working with hazardous chemicals.
7. Be familiar about the working of instrument prior to handling it independently. Keep all the laboratory equipments at their respective places after use.
8. Do not remove any culture or any other article from the laboratory.
9. In case of any accident or spillage of culture and stain, immediately report to the instructor or laboratory in-charge for its proper care and disposal.
10. Always, discard the disposables and used cotton, matchstick, paper pieces etc. to trash cans and never into the sink.
11. Always keep disinfectant solution jar on the working bench for disposal of refuse.
12. Do not bring and consume eatables in the laboratory.
13. Dispose of infectious material, cultures and contaminated material carefully in container of disinfectant and check its disinfecting activity regularly.
14. Dispose of all cultures after autoclaving and used pipettes should be placed in disinfectant after use.
15. Always flame the inoculating loops before and after use. Similarly sterilize the necks of all tubes and flasks by passing it through the burner flame before and after each operation.
16. Arrange the cultures and bench equipment at respective place on the desk. Do not allow unused articles to accumulate in your work area.
17. Always, follow the instructions sincerely.
18. Work either using laminar flow chamber or light the burner at least five min prior to making any inoculations and work near the burner.
19. Avoid horseplay and vocalization in the laboratory

Unit one
Observations and study of structure of microbes

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Exercise 1: Demonstration of omnipresence of microbes

Microorganisms are present everywhere in the universe. They are part and parcel of our life. These are **omnipresent** i.e. present in air we breathe, food we eat and water we drink as well as on our body surface. Some of these comprise the resident flora of skin and mucosal surfaces of our body that are in contact with the atmosphere. Distribution and composition of microflora at particular niches depend on the prevailing environmental conditions and availability of nutrients. It is very difficult to dislodge them from these places. They colonize quickly if removed deliberately using cleansing devices, e.g. washing of hands with simple water or soap. However, scrubbing does decrease their number temporarily. It took very long to prove the existence of these microscopic forms until the developments of microscope and culturing techniques of these organisms. Presence of these organisms can be demonstrated by fingerprinting experiment. Each organism transferred to nutrient medium grows and produces a visible growth called colony representing the progeny of single organism. Number of such colonies appearing on nutrient medium on incubation decreases as the same finger is touched again and again at different places subsequently.

Requirements

- a. Nutrient agar plates
- b. Soap or any other detergent, alcohol
- c. Incubator.

Procedure

1. Take a nutrient agar plate. Remove its lid near the Bunsen burner flame. Now, touch the agar surface at 5-6 places with the forefinger.
2. Repeat the same protocol on second plate with soap or alcohol washed hands.
3. Incubate both the plates in inverted position in the incubator at 37°C.
4. Next day observe both the plates and note the size and number of colonies in each fingerprint impression.
5. Study the colonial morphology of different types of colonies in term of size, shape, color, consistency, translucency, elevation etc. Keep these plates to be used to study the morphology of organisms in next period.

Questions

1. What do you mean by a colony?
2. What is the source of organisms found in air?
3. Why are the plates incubated in inverted position?
4. How did the invention of agar helped in development of microbiology?
5. Name the procedures used in the study of bacteria.

Exercise 2: Microscopy: Study, use and care of microscope

Microscope is an important tool for the microbiologist as the microorganisms are invisible to naked eye unless magnified. Antony van Leeuwenhoek called as “the father of microbiology” depicted the drawings of major forms of microorganisms while looking through a magnifying glass mounted on mechanical device for observing microbes. It was called a **simple microscope**. It contains a biconvex lens whose movement could be mechanically regulated.

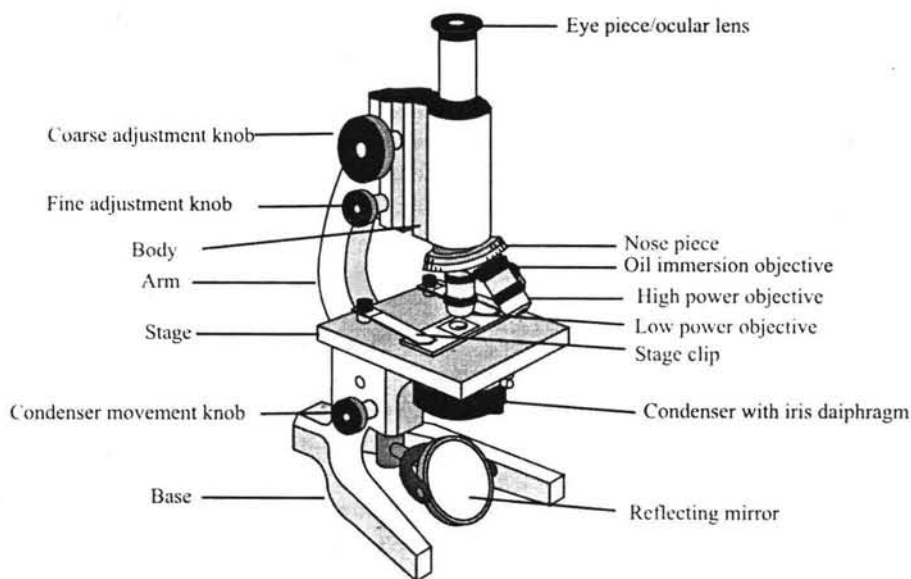
Bright field microscope, a compound microscope, incorporates two or more lens system. The arrangement increases the net magnification of the object many folds depending upon the magnification of two lenses. Zacharias Jansen introduced microscope currently used in most laboratories. Quality of microscope depends on its resolution or resolving power of the objective lens. Microscope comprises two main parts, the supporting stand and the optical system.

The supporting stand includes: (1) a **base** to hold the microscope in its position, (2) an **arm** to support the optical system and house the fine adjustment, (3) a **stage** or **platform** on which the object to be examined is placed. It is usually equipped with mechanical device that holds the glass slide firmly and on which the object is mounted so that it can be moved from place to place by setscrews. (4) **Condenser and mirror** is fitted beneath the stage. Condenser and mirror focus the light through a central opening in the stage on the object to be examined.

The optical system consists of a body tube, which supports ocular lens (eye piece) at top and a set of objective lenses attached to revolving nose piece at the other end. Optical system is connected to the arm of the supporting stand by an intermediate slide, which moves up and down on the arm in response to movement of fine adjustment. The intermediate slide contains the rack and pinion for the coarse adjustment, which acts directly on the tube of the optical system. **Iris diaphragm** controls the intensity of light entering the object through condenser. **Ocular lens** (5x, 10x or 15x) is placed at the upper end of the tube. Monocular microscope has one-lens while the binocular has two lenses. **Set of objective lenses** (10x, 40x and 100x an oil immersion objective): Above the stage on other end of mechanical tube is a revolving nosepiece holding three/four objectives. These primary lenses magnify the specimen. The objective can be moved away or closer to the object through **coarse** (low power objective) and **fine adjustment knobs** (for high power and oil immersion objective) for focusing the image. **Reflecting mirror/ light source**: Reflecting mirror has two planes, one concave for incandescent or artificial light and other plane for daylight.

Magnification of microscope is the multiplication product of the powers of objective and ocular lenses. Magnification of an objective is usually designated by its equivalent focal length (the focal distance/ working distance of a lens). 16 mm objective magnifies 10 times; 4 mm objective magnifies 40-45 times and most 1.8 mm objectives 97 times.

A **phase contrast microscope** converts slight differences in refractive index and density into easily detectable variations in light intensity and is an excellent device to observe living cells, as there are little differences in contrast between the cells and water. **Microscope condenser** has an annular stop and an opaque disk with a thin transparent ring which produces a hollow cone of light so that background formed by non-deviated light is bright while the unstained object appears dark as the light passing through cone/cell. This type of microscope is highly useful for the detection of bacterial components such as endospores and inclusion bodies containing polyβ hydroxybutyrate, polyphosphates, sulfur or other substances.



Compound light microscope

Fluorescence microscope : Fluorescence is the property of emitting rays having wavelength different from that of incident rays. Object becomes luminous against dark background. Fluorescent materials are generally of two kinds: one present naturally in cells and the other include the induced one by staining the object with fluorescent dyes or flurochromes. In fluorescent microscope, an object emits light when examined under UV rays. Radiations exciting the luminosity do not contribute to image formation. Such objects absorb radiant energy and release trapped energy when excited as visible light quickly to return to more stable state. Two kinds of filters are used for filtering harmful rays. **Excitation filters**, which transmit only the rays of visible range while blocking UV radiations the illuminating beam only excluding the radiations, and **Barrier filters** prevent passing of excited radiations in microscope to protect eyes from UV rays. The technique has become very important in medical microbiology, microbial ecology and study of bacterial pathogenesis. The objects are identified after staining with fluorescent dyes or flurochromes or specifically labeled fluorescent antibodies using immunofluorescence procedures.

Handling and use of microscope

1. Always, carry the microscope with both hands, one beneath and other on the arm.
2. Always focus the object by moving the *Objective* away from the glass slide. Avoid focusing downwards.
3. With coarse adjustment knob move the Objective to be used until it nearly touches the surface upper surface of the mounted specimen. Then focus by moving the coarse knob

upwards until the object comes into view. Complete the focusing with Fine adjustment knob.

4. Adjust the mirror and light while using low power Objective to give adequate illumination.
5. While observing unstained objects, the *Iris diaphragm* should be barely open to achieve good contrast. Iris diaphragm is fully open with higher magnification and while viewing stained objects.
6. Always, clean the lenses before and after use with lens paper. Do not touch the lenses with hands. Leave *Objectives* with lowest power in working position.
7. Keep the microscope covered when not in use.
8. Observe the slides with both eyes open. Do not incline the microscope. Adjust the stool to use the instrument comfortably.
9. Avoid direct sunlight. North light is advantageous.
10. To achieve a good contrast adjust the *Iris diaphragm*. Usually the Iris diaphragm is open to the minimal level while examining the unstained objects and it is fully open when stained specimens are examined. Observe the change in specimen when the Iris diaphragm is opened or closed for obtaining good contrast.
11. Place a drop of immersion oil on the illuminated area of slide and shift to 100X objective. Lower the *Objective* slowly viewing from sides until the lens contacts the oil drop and then the surface of slide.
12. Prior you place the microscope in wooden box at the conclusion of each laboratory period turn the nosepiece until the low power objective is in place and lower to the maximum until it reaches stop.

Questions

1. Which objective focuses closest to the object?
2. What controls the light entering the *Ocular lens*?
3. What is false and useful magnification?
4. What is field of vision?
5. How can you enhance the resolving power of microscope?
6. What would occur if you use water instead of immersion oil?
7. Examine a drop of urine sediment under microscope and make sketch showing different objects seen.
8. What is "working distance" in regard to compound microscope?
9. How would you distinguish dust particle and microorganisms?

Exercise 3: Examination of microorganisms in live preparations.

- i. Hay infusion examination.**
- ii. Examination of protozoa**
- iii. Hanging drop technique**
- iv. Motility in semi solid agar.**

Microorganisms can be divided into two broad groups based on their cell structure: the prokaryotes and eucaryotes. The former lack nucleus and several other membranes bound organelles include bacteria and blue-green algae or the cyanobacteria. Fungi, algae, protozoa and the multicellular parasites are some examples of eucaryotes. Staining procedures used for examining microorganisms are usually harsh and often distort the natural structure, shape and formations. Bacteria can be observed under a microscope in unstained or stained preparation. Even the motility in bacteria can be observed in live preparations using wet preparations. A wet mount is the fast way to observe bacteria. In hanging drop technique the application of petroleum jelly seal around the cavity reduces the evaporation of suspended fluid drop. Making it possible to observe larger microbes and motile organisms more easily because of greater depth provided by the hanging drop. Motility of bacteria can also detected by observing the growth pattern of culture in semisolid or motility agar inoculated with a straight wire on overnight incubation.

Requirements

- a. Hay infusion or pond water
- b. Microscopic slide
- c. Cavity slide, cover slip petroleum jelly, matchstick
- d. Inoculating wire
- e. Motility agar or Hugh Leifson medium in sugar tubes
- f. Bacterial culture: *Proteus vulgaris* broth culture (8-16 h old).

Procedure

(i) Hay infusion or pond water examination

1. Take a drop of hay infusion or pond water on clean grease free glass slide.
2. Place a cover slip on the drop from the edge to avoid air bubbles entrapment.
3. Focus the slide under microscope, with condenser lowered to maximum and iris opening narrowed down. Examine the slide under microscope initially under 10x objective and then shift to 40x objective.
4. Record the observation regarding various forms of organisms seen differing in cell structure, motility, size, shape, color etc and illustrate these diagrammatically.

(ii) Examination of protozoa

The protozoa can be seen more distinctly in unstained condition as compared to bacteria under bright field microscope. The protozoa are larger as compared to bacteria. In some, even the internal structures can easily be observed. Morphology of protozoa is usually diagnostic in many cases. The group display different types of reproduction activities rather than merely dividing by binary fission seen like bacteria.

Requirements

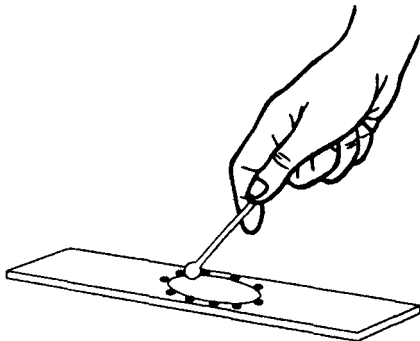
- a. Hay infusion
- b. 3% gelatin solution
- c. Plastic dropping pipette

Procedure

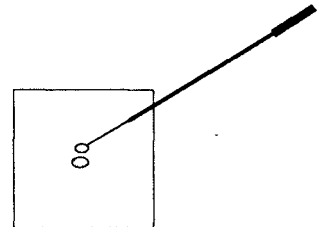
1. Allow the melted gelatin solution to cool to room temperature
2. Mix one drop of gelatin and one drop of hay infusion on the microscopic slide.
3. Place cover slip and examine under microscope.
4. Note down the morphology observed under different objectives (10x, 40x and 100x) and sketch the observations.

(iii) Hanging drop technique

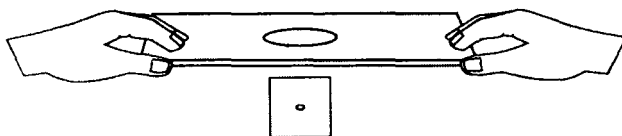
1. Take a clean cavity slide. Apply petroleum jelly around the well with matchstick.
2. Pick up a cover slip and place it on filter paper. Transfer a loopful culture in the center of cover slip.
3. Now, carefully put the slide over the cover slip so that drop is in the center of cavity. Carefully invert the slide so that drop hangs in the well.
4. Examine the slide under low power objective. Locate the edge of the drop by moving the slide with pinion arrangement and position the slide so that the edge of the drop crosses the center of the field.
5. Reduce the light adjusting iris diaphragm and condenser and focus. Observe the different sizes, shapes, and types of movement.



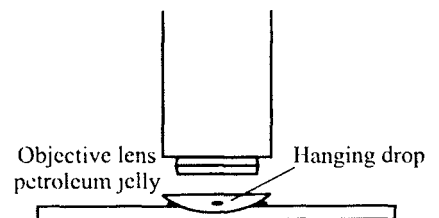
(I) Apply petroleum jelly around the well



(II) Place a drop of culture in the center of cover slip



(III) Invert the cavity slide with well facing the culture drop on cavity slide and gently press it and turn up slide



(IV) Observe under microscope

Hanging drop technique

(iv) Motility in semisolid agar

1. Aseptically inoculate the sterile semi solid medium in tube by stabbing with a straight inoculating needle.
2. Incubate the inoculated tubes at 37°C for overnight.
3. Next day examine the semi solid agar tubes for bacterial growth pattern of culture.
4. The culture is non motile if the growth is restricted to the inoculated path and motile if the tube shows turbidity diffusing along the streak or throughout the medium.

Questions

1. Why are microorganisms hard to see in wet preparations?
2. Why the oil immersion lens is not used in examination of hanging drop procedure?
3. Why did you use young cultures for observing bacterial movement?
4. What is the motility component in bacteria?
5. Why do we add gelatin prior to observing under microscope?
6. What would be the effect of culture shaking on bacterial motility when examined in hanging drop?
7. Enlist the similarities and differences between protozoa and algae.
8. What is the purpose of a hanging-drop preparation?

Exercise 4: Examination of blue green algae (BGA)

Blue green algae are found throughout world in fresh water and saltwater and in moist microaerophilic habitats. These are abundant in fresh water near the surface than deep in water or in saline habitats. Accumulation of water for prolonged periods under sunny habitats is usually good for algal growth as these conditions favor the development of blue green algae (BGA), which have become dormant during dry seasons. Red sea has been so named due to the abundance of red BGA. Most algae are forage plants or planktons for fishes and other sea animals. However the presence of BGA in drinking water is discouraged as they impart disagreeable smell, odors and taste and some may cause bloom. Some are even rich source of vitamins and minerals.

Requirements

- a. Prepared slides of mixed BGA
- b. Water sample from shady pond

Procedure

1. Compare the morphology of algal cells on the reference slides.
2. Sketch and label the different parts of the cells seen on each slide.
3. Place a drop of pond water on the slide and cover it with the cover slip.
4. Examine care fully and try to identify the type of algae present and note down their movement. Sketch the different types of cells seen and the movements.

Questions

1. What is the economic importance of BGA?
2. What are planktons?
3. What disease or nuisance does BGA cause to mankind?
4. Compare and contrast the properties of BGA and bacteria.

Exercise 5 : Preparation of bacterial smear and simple staining

Microorganisms are small, transparent and motile in fluids hence it is very difficult to observe under microscope unless these are immobilized (fixed) and stained with a suitable stain. Placing and spreading of sample on microscopic slide is called a **smear**. The air-dried smear is fixed to glass to avoid it being washed when treated with liquid stain.

Fixation is accomplished by passing the smear through the top part of Bunsen burner flame 2-4 times taking care that glass slide is quite hot but bearable to the reverse of your hand skin. Alternatively the smear can be chemically fixed by covering the smear with 95% methanol for 1-5 min. **Fixation** denatures the bacterial enzymes and prevents autolysis and ensures bacterial adherence to microscopic slide. Soon after fixing, slide can be stained.

In bacteriology, three classes of stains are used: simple stain, differential stain and special stain or structural stain. Simple stain is usually an aqueous or alcoholic solution of dye applied 1-2 min to the smear and then washed off. Most common simple stains are Loeffler's alkaline methylene blue, methylene blue, safranin, carbol fuchsin and gentian violet. Most bacteria stain easily and quickly with simple stains while the capsules and spores remain unstained.

Most stains used in microbiology laboratory are the aniline dyes. The ion imparting color to the dye is called chromophore. The dyes are usually salts, a few are acids or bases composed of colored ions. If chromophore is a positive ion like methylene blue, it is called a basic stain and if negative, it is known as acidic stain.

Methylene blue chloride $\longrightarrow$ methylene blue (chromophore) + chloride

Staining procedure that uses only one stain is called simple staining. Simple stain that stains bacteria is direct stain and which does not stain bacteria but stains background is called negative staining. Simple staining is easy, cost effective and very useful in studying the morphology, size and arrangement of microorganisms.

Requirements

- Stains: Methylene blue, safranin, malachite green or diluted carbol fuchsin solution.
- Bacterial cultures: *Staphylococcus epidermidis* slant and *Bacillus megaterium* and *Escherichia coli* broth.

Other documents randomly have
different content

Dragomira pâlit un moment. Mais elle eut bientôt recouvré son calme et sa décision.

"Fuis! dit-elle d'un ton de commandement énergique, votre affaire est de continuer l'oeuvre sainte! Sauvez-vous!

- Je reste avec toi, s'écria Henryka.

- Non, fuis, je te l'ordonne, en toute hâte, à cheval! Je reste ici pour juger au nom du Tout-Puissant!"

Henryka se jeta dans les bras de Dragomira et lui donna un baiser; puis elle sortit rapidement, sauta sur le cheval de Zésim et partit au galop. Karow et Tabisch se sauvèrent par le jardin, passèrent par-dessus la clôture de planches et disparurent dans la forêt.

Dragomira prit son revolver et attendit Anitta de sang-froid.

On entendit le trépignement des pieds des chevaux, des pas lourds, le cliquetis des armes, une voix claire qui donnait des ordres. Puis le silence se fit, et Anitta entra, accompagnée de Tarass. Elle portait, elle aussi, la jupe courte, les bottes d'homme, la pelisse de mouton et le mouchoir de tête d'une paysanne petite-russienne. Elle avait un pistolet à la main; Tarass était armé d'un fusil de chasse.

"Rends-toi, scélérate! cria Anitta; le cabaret est entouré par mes gens. Tu es entre mes mains. Tu ne peux t'échapper."

Dragomira dressa fièrement la tête.

"Je t'ai attendue, répondit-elle, pour régler mon compte avec toi. Cette heure est celle du châtement que je veux vous infliger au nom

de Dieu, à toi et à celui qui est là!

- Tu blasphèmes Dieu quand tu prononces son nom, dit Anitta, il a horreur de toi et de ta doctrine homicide.

- Dieu décidera entre toi et moi.

- Qu'il décide! répondit Anitta, regardant avec calme son ennemie bien en face, nous sommes toutes les deux devant le Juge éternel. Qu'il prononce!"

Un sourire triomphant passa sur le beau et fier visage de la Pêcheuse d'âmes, pendant qu'Anitta faisait à voix basse une courte prière.

Toutes les deux levèrent en même temps leurs pistolets. Il y eut un instant d'anxieuse attente, puis Dragomira pressa la détente.

Le coup ne partit pas.

L'autre chien s'abattit. On vit un éclair, on entendit une détonation. Dragomira fit encore un pas vers Anitta et tomba tout à coup en avant le visage contre terre.

"Est-elle morte?" demanda Anitta.

Tarass s'approcha de Dragomira et la retourna:

"Dieu a jugé, dit-il, son âme est devant lui."

Anitta se mit à genoux et éleva en pleurant ses bras vers le ciel. Puis elle se releva, tira le poignard qu'elle portait à sa ceinture, coupa rapidement les cordes qui liaient son bien-aimé, et, sanglotant de joie, le serra contre sa poitrine.

"Sauvé! murmura Zésim, sauvé par toi!"

La nourrice se précipita alors dans la salle, et se suspendit en pleurant au cou de Zésim.

"Mon enfant! s'écria-t-elle, mon cher enfant! Le ciel t'a protégé et cet ange t'a sauvé!"

Le traîneau de Kachna fut bientôt attelé. Zésim aida Anitta à y monter, et Tarass sauta sur le siège du cocher. On partit au galop pour Kiew et l'on alla droit au palais Oginski.

Zésim, tout triomphant, rendit la bien-aimée à son père et à sa mère, qui bénirent le jeune couple en versant des larmes de joie et rendirent grâces à Dieu par un vœu solennel.

Aujourd'hui, à Kasinka Mala, à la place où était jadis le cabaret et où Dragomira mourut, s'élève une chapelle dédiée à la Vierge. Tous les ans, au jour anniversaire de celui où Zésim fut sauvé par Anitta d'une façon si merveilleuse, un prêtre y dit une messe basse pour l'âme de la malheureuse, victime d'une épouvantable superstition.

FIN

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